



TITLE:

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# **Erythromycin Treatment Suppresses Myocardial Injury in Autoimmune Myocarditis in Rats via Suppression of Superoxide Production**

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## Abstract

**Background.** Recent evidence suggests that erythromycin (EM), a major macrolide antibiotic, has many biological functions in addition to the anti-bacterial actions, including anti-inflammatory and free radical scavenging actions. However, the effects of the drug upon inflammatory myocardial diseases are unknown. We tested the hypothesis that EM ameliorates experimental autoimmune myocarditis in rats attributing to the suppression of superoxide production. **Methods.** We administered EM, 3.3mg/kg/day and 33mg/kg/day, intraperitoneally for 3 weeks, to rats with experimental autoimmune myocarditis (EAM) produced by immunization by porcine myosin. **Results.** EM treatment reduced the severity of myocarditis compared with the untreated group in a dose-dependent manner by comparing the heart weight/body weight ratio, pathologic scores, and myocardial macrophage, CD4<sup>+</sup>, and CD8<sup>+</sup> infiltrations. Echocardiographic study showed that increased left ventricular posterior wall thickness produced by myocardial inflammation was reduced by EM treatment. Myocardial superoxide production, determined by dihydroethidium staining, was significantly reduced by the treatment. Western blotting showed that the expression of myocardial interleukin-1 $\beta$  was reduced by EM treatment compared with untreated groups. The in vivo dorsal air pouch model showed that EM significantly suppressed leukocyte chemotaxis in a dose-dependent manner. **Conclusion.** Irrespective of a well-known classic antibiotic, EM attenuated EAM not only by the anti-inflammatory action but by the suppression of superoxide production.

**Keywords:** autoimmune myocarditis; erythromycin; 14- member ring macrolides; superoxide production

## 1. Introduction

In humans, acute myocarditis is a potentially lethal disease, and frequently precedes the development of dilated cardiomyopathy (DCM). In general, two major mechanisms to explain how myocarditis develops into DCM have been proposed; one is a persistent antigen existence, and the other is a progressive autoimmune myocardial injury <sup>(1)</sup>. The autoimmune giant cell myocarditis in rats, i.e., experimental autoimmune myocarditis (EAM), mimics human fulminant myocarditis in the acute phase <sup>(2)</sup>.

Erythromycin (EM) is known as a classic 14-member ring macrolide and a potent antibiotic for the treatment of various microbial infections <sup>(3)</sup>. Recently, macrolides, including EM, have been reported to have multiple biologic effects, such as alteration of inflammatory and free radical actions <sup>(4-6)</sup>. It was demonstrated that free radicals play an important role in the development of heart failure in this animal model <sup>(7)</sup>. However, the effects of EM upon inflammatory myocardial diseases are still unknown.

The purpose of the present study was to examine the effects of EM upon an EAM model, focusing on its inhibitory effects on the production of inflammatory cytokines and free radicals.

## 2. Materials and Methods

### 2.1. Immunization

EAM was induced in Lewis rats by immunization with porcine cardiac myosin as previously described <sup>(8-10)</sup>. Porcine cardiac myosin (Sigma) was injected subcutaneously in the foot pads with 0.1 ml of myosin (10 mg/ml) mixed with an equal volume of Freund's complete adjuvant (FCA) supplemented with *Mycobacterium tuberculosis*

H37Ra (Difco) on days 1 and 8. Control rats were immunized with FCA alone. The animals were daily observed up to the end of the experiment on day 21. The day of injection was designated day 1.

Rats surviving 21 days after myosin or FCA immunization were used for the cardiac function study, and thereafter were sacrificed.

## 2.2 Medication

Rats with EAM were divided into three groups and treated intraperitoneally with phosphate buffered saline (PBS) (untreated group, n=15) and EM (3.3mg/kg/day, n=15 and 33mg/kg/day, n=10) for three weeks (immunized protocol). These doses were chosen because of the previous studies <sup>(4-6)</sup>. In parallel with the protocol, additional control groups (unimmunized protocol) were age-matched unimmunized rats treated for 21 days with PBS (untreated group, n=5) and EM (3.3mg/kg/day, n=5 and 33mg/kg/day, n=5). All the mice were sacrificed three weeks thereafter. Organ weights were determined. Organs including the skins at dorsal air pouch were processed for the pathologic study. The animals were sacrificed under the light ether anesthesia at the end of experiments.

We performed animal experiments in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996), and these were approved by the institutional ethics committee for animal experiments of Kyoto University.

## 2.3. Histopathology

At sacrifice, macroscopic findings were performed. Microscopic findings for

myocardial necrosis and cellular infiltration were graded on a scale of 0 to +5, as previously described<sup>(8-10)</sup>. That is, myocardial sections were graded by two of the authors (K.S., C.K.) who had no knowledge of the respective treatment groups on a scale of 1+ (mild) to 5+ (very severe) for the severity<sup>(8-10)</sup>. After macroscopic examination, a part of the ventricles was embedded in OCT for immunohistochemistry.

#### **2.4. Immunohistochemical staining**

We used an immunoperoxidase technique to perform immunohistochemistry for cell surface markers, as previously described<sup>(8-10)</sup>. In brief, anti-macrophage (anti-M $\phi$ , ED1, 1:400, PharMingen), anti-CD4 (GK1.5, 1:50, PharMingen), and anti-CD8 (53-6.7, 1:50, PharMingen) antibodies were applied to acetone-fixed cryosections. After being washed, the sections were then exposed to a second antibody (horseradish peroxidase-conjugated antibodies), and the antibody was visualized with diaminobenzidine. Sections were counterstained with 1% methyl green. The positive-staining cells of heart tissue were counted blindly by two observers in six fields at  $\times 400$  magnification (within a 1-mm<sup>2</sup> grid), and the total positive-staining cells of the six fields were recorded as the number of infiltrating cells in the lesions. The percentage of stained cell was then calculated for each antibody staining section<sup>(8,11)</sup>.

#### **2.5. In situ detection of superoxide production in hearts**

To evaluate in situ superoxide production from hearts, unfixed frozen cross sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method<sup>(11-14)</sup>. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by

intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10- $\mu$ m thick sections, and incubated with 10  $\mu$ M DHE at 37 C° for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope. Superoxide production was demonstrated by red fluorescence labeling.

For quantification of ethidium fluorescence from hearts, fluorescence (intensity x area) was measured in five randomly selected fields using the high-power image monitor.

## 2.6. Western blotting

Western blot analysis for interleukin-1 $\beta$  (IL-1 $\beta$ ) was performed as described previously <sup>(7,9,10)</sup>. The myocardial lysates were electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were sequentially electrophoretically transferred to a membrane (Millipore). The membrane was incubated with an anti-IL-1 $\beta$  antibody (Serotec) and then with a peroxidase-linked secondary antibody (Amersham). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) samples were probed in the blots as internal controls for loading. Resulting bands were quantified as optical density band area by the image analysis system.

## 2.7. Echocardiographic and hemodynamic studies

Echocardiography was performed on day 21 post-myosin injection for the subpopulation of control (immunized and unimmunized) and EM (33mg/kg/day, immunized and unimmunized) groups. Rats were anesthetized with an intraperitoneal injection of 0.040 to 0.060 mg/mg sodium pentobarbital. A 12 MHz probe was placed at the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos

5500, Philips). Left ventricular (LV) end-diastolic dimension (LVDd), left ventricular end-systolic dimension (LVDs), interventricular septum (IVS) thickness, and posterior wall (PW) thickness were measured, as an average of three beats. Ejection fraction (EF) of the left ventricle was calculated<sup>(10, 11)</sup>.

## 2.8. Chemotaxis in vivo

A dorsal air pouch was created as previously described<sup>(15)</sup>. Briefly, rats were injected on the back subcutaneously with 4 ml of air under the anesthesia of sodium pentobarbital on day 10. PBS (0.3ml) was injected into the air pouch. Air pouch was maintained up to day 20. The air pouch was flushed quantitatively with PBS, and four hours later, the volume recovered was measured. The number of cells was determined with a hemocytometer. The exuding cell numbers into the air pouch on day 20 were calculated. The cutaneous tissues at the dorsal air pouch were pathologically examined.

## 2.9. Statistical analysis

All values were expressed as means±standard deviation (SD). One way analysis of variance (ANOVA), following by Fisher protected least significant difference test, was performed. A value of  $P<0.05$  was considered statistically significant.

# 3. Results

## 3.1. General clinical findings

No rats died in all groups during three weeks of the study.

## 3.2. Histopathology and organ weights in rats with acute EAM (Tables 1, 2)

Extensive injuries to myocytes with inflammatory changes and multinucleated giant cells associated with ED1 (macrophage) expression were observed (Figure 1).



Treatment with EM 33mg/kg/day, but not with EM 3.3mg/kg/day, reduced the severity of the disease (Figure 1), as assessed by measuring heart weight/body weight (HW/BW) and microscopic scores.

The percentages of ED1<sup>+</sup>(macrophage-positive), CD4<sup>+</sup>, and CD8<sup>+</sup> T cells recruited into the lesions were significantly reduced by the treatment of EM (Figure 1). No positive cells were found in the unimmunized groups.

The dorsal subcutaneous tissue was swollen by the production of air pouch compared with the normal tissue. However, the treatment of EM clearly suppressed the thickness of the subcutaneous tissue swelling compared with that of untreated mice (Figure 2).

### **3.3. In situ superoxide production (Table 2)**

Ethidium fluorescence was detected from the myocardial lesions, and EM treatment significantly reduced the intensity of the staining (Figure 1). That is, by the treatment of EM, superoxide production was significantly decreased compared with the control.

### **3.4. Effects of EM on IL-1 $\beta$ expression**

As shown in Figure 3, inhibitory effect of EM against the expression of myocardial IL-1 $\beta$  was demonstrated by the Western blotting.

### **3.5. Cardiac function (Table 3, Figure 4)**

After 3 weeks post-myosin injection, EF tended to slightly decrease in rats with myocarditis compared with those of control rats. EM administration did not change cardiac function significantly; i.e., the treatment did not produce an increase in EF or a

decrease in LVDd compared with the untreated group. Namely, the significant improvement of cardiac function compared with the untreated rats was not observed in the rats treated with EM. However, EM treatment suppressed the increase of LVPW thickness compared with the untreated groups. The increased LVPW at this stage is considered to be due to interstitial edema caused by severe myocardial injury.<sup>(10,11)</sup>

### 3.6. Effects of EM on chemotaxis (Table 2)

The number of leukocytes exudated in the air pouch on day 20 was suppressed by EM administration in a dose-dependent manner.

## 4. Discussion

EM is a classic and potent antibiotic with a 14-member ring macrolide for the treatment of various microbial infections, and has multiple biologic effects<sup>(3-6)</sup>. In this report, we have demonstrated for the first time that EM inhibited expression of various inflammatory cells in the myocardium and suppressed EAM in rats associated with decreased superoxide production.

Several studies have described the participation of cytokine overproduction in the pathogenesis of heart failure<sup>(16-18)</sup>. Recent reports indicated that myocardial injury was produced by inflammatory cytokines, and that lipid peroxidation was caused by them, resulting in cell membrane damage in heart failure<sup>(19)</sup>. Several studies have definitely established the key role of the Th1 cytokine system in the pathogenesis of heart failure by demonstrating that agents that inhibit the generation of Th1 cytokine confer cardiovascular benefit<sup>(20, 21)</sup>.

In the present study, the immunohistochemical study showed that the numbers of

macrophages, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were markedly reduced by EM treatment. Accordingly, it may be that the main beneficial effects of EM upon EAM may be partly due to the suppression of inflammatory events in the myocardium. Indeed it was confirmed in the current study that EM administration suppressed the myocardial expression of IL-1 $\beta$ . The molecular mechanisms of EM for the suppression of inflammatory cytokines are not fully understood. However, the inhibition of signal transduction pathways, such as NF- $\kappa$ B pathway, which is particularly important in the regulation of cytokines, has been postulated<sup>(22-24)</sup>.

Next, EM suppressed the generation of free radicals in rats with myocarditis. As a result, it may be that EM treatment protected against superoxide-mediated oxidative damage in vivo. It was demonstrated that free radicals play an important role in the development of heart failure in this animal model<sup>(7, 11)</sup>. Accordingly, EM treatment may act for reducing superoxide production, resulting in less severe myocardial damage in rats with myocarditis.

Whether EM directly affects the host immune status or not is unknown, it was demonstrated, however, in the air pouch study that the numbers of inflammatory cells in rats immunized with myosin treated with EM was reduced compared with those immunized with myosin and treated with PBS. Furthermore, EM treatment suppressed the subcutaneous tissue swelling at air pouch sites, indicating the indirect evidence of anti-chemotoxic activity of the drug. Accordingly, the overall effects of the drug could induce the suppression of autoimmune myocarditis associated with the reduction of chemotaxis activity.

It was already reported that clarithromycin, another new 14-member ring macrolide, has the potential to suppress cardiac rejection by the suppression of matrix metalloproteinase in a murine cardiac transplantation model <sup>(23)</sup>. Recently, it has been reported that tetracycline and its derivatives demonstrate the suppressive activity of matrix metalloproteinase in a rabbit model of atherosclerosis <sup>(25)</sup>. In addition, tetracyclines also inhibit production of inflammatory cytokines and free radicals, and have been used effectively in acute lung injury and chronic inflammatory diseases <sup>(26, 27)</sup>. Most recently, Hishikari et al. <sup>(28)</sup> reported the beneficial effects of early treatment of clarithromycin upon EAM; they showed that early clarithromycin treatment is effective to attenuate myocarditis by suppressing matrix metalloproteinase-9 activity.

The lack of the improvement of cardiac function may be due at least in part to the characteristic of this model, i.e., the inflammatory process continues until 3 weeks and thereafter decreases and then ventricular remodeling begins; the interventricular septal and LV posterior wall thickening might be due to the inflammatory changes of the myocardium during the acute stage of EAM. As a result, EM exhibited the antihypertrophic effect against increased LV mass alone.

EM itself is a classic antibiotic agent. From our data, however, EM treatment might be promising as a new therapy for heart failure, particularly for ventricular remodeling where ongoing autoimmune process may play a role in the disease development. In conclusion, EM ameliorates EAM in rats. The cardioprotection of EM may be due to the suppression of not only inflammation but superoxide production.

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**Conflict of interest:** none declared.

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## Figure Legends

### Figure 1. Pathological findings, DHE stainings, and immunohistological findings

Severe myocardial necrosis with cellular infiltrates (black arrows) and massive superoxide production (white arrows) was seen in the untreated rat with myocarditis (upper pannel), but less severe myocardial necrosis with cellular infiltrates was noted in the EM (33mg/kg/day) -treated rat with myocarditis (lower panel). Serial sections were used in both groups. Arrows in the magnified insets ( $\times 480$ ) indicate giant cells.

CD4, helper T stain

CD8, suppressor T stain

DHE, dihydroethidium

ED1, macrophage stain

HE, hematoxylin-eosin

IH, immunohistology

( $\times 230$ )

### Figure 2. Cutaneous tissue swelling at air pouch site

The subcutaneous tissue (B, C, D, E) was swollen by the production of air pouch compared with the normal (A). However, the treatment of EM (33mg/kg/day) (C, E) clearly suppressed the thickness of the subcutaneous tissue swelling compared with that of untreated mice (B, D).

Hook-shaped width indicates the degree of subcutaneous tissue swelling.

A; Normal

B; myosin-unimmunized, untreated

C; myosin-unimmunized, EM-treated

D; myosin-immunized, untreated

E; myosin-immunized, EM-treated

HE (× 230)

Figure 3. Myocardial IL-1 $\beta$  expression

(A) Representative Western blot analysis showed the decreased expression of IL-1 $\beta$  in EM (33mg/kg/day)-treated rats with myocarditis compared with untreated rats with myocarditis. (B) Densitometric analysis of relative protein levels showed that IL-1 $\beta$  expression was increased in rats with myocarditis, but was decreased by EM treatment. Values were derived from 5 animals and represent a percentage of controls. Lanes were designated as C, control (myosin-unimmunized, untreated) rat; EM, myosin-unimmunized, EM (33mg/kg/day)-treated rat; M, myocarditis (myosin-immunized, untreated) rat; M+EM, myosin-immunized, EM (33mg/kg/day)-treated rat. \*\*P<0.01 vs M, † P<0.01 vs C.

Figure 4. Echocardiographic findings

The significant improvement of cardiac function compared with the untreated rats was not observed in the rats treated with EM (33mg/kg/day). However, EM treatment (C) suppressed the increase of LVPW (white hook-shaped width) compared with the untreated group (B).

A; myosin-unimmunized, untreated

B; myosin-immunized, untreated

C; myosin-immunized, EM-treated

Figure 1

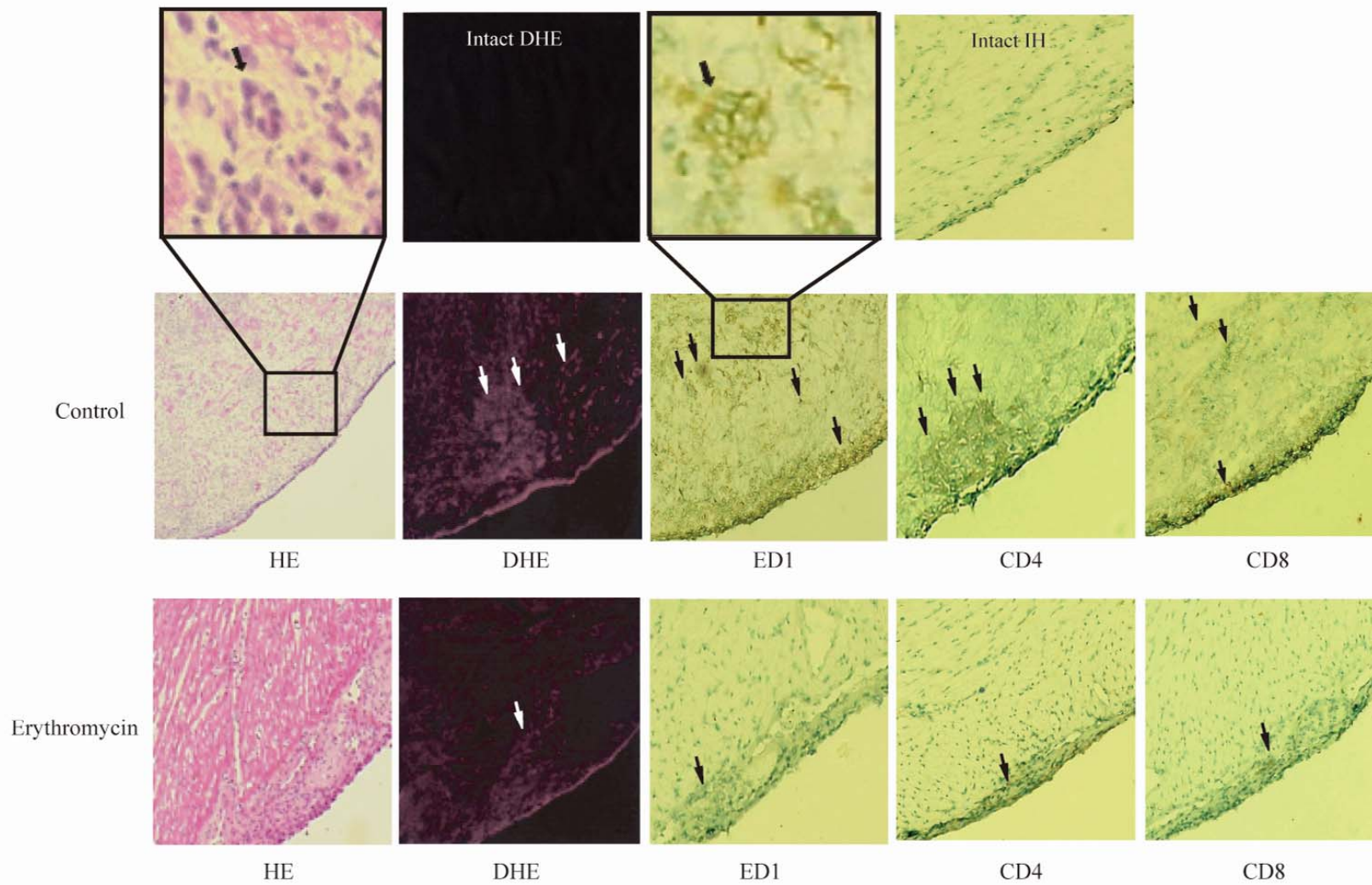


Figure 2

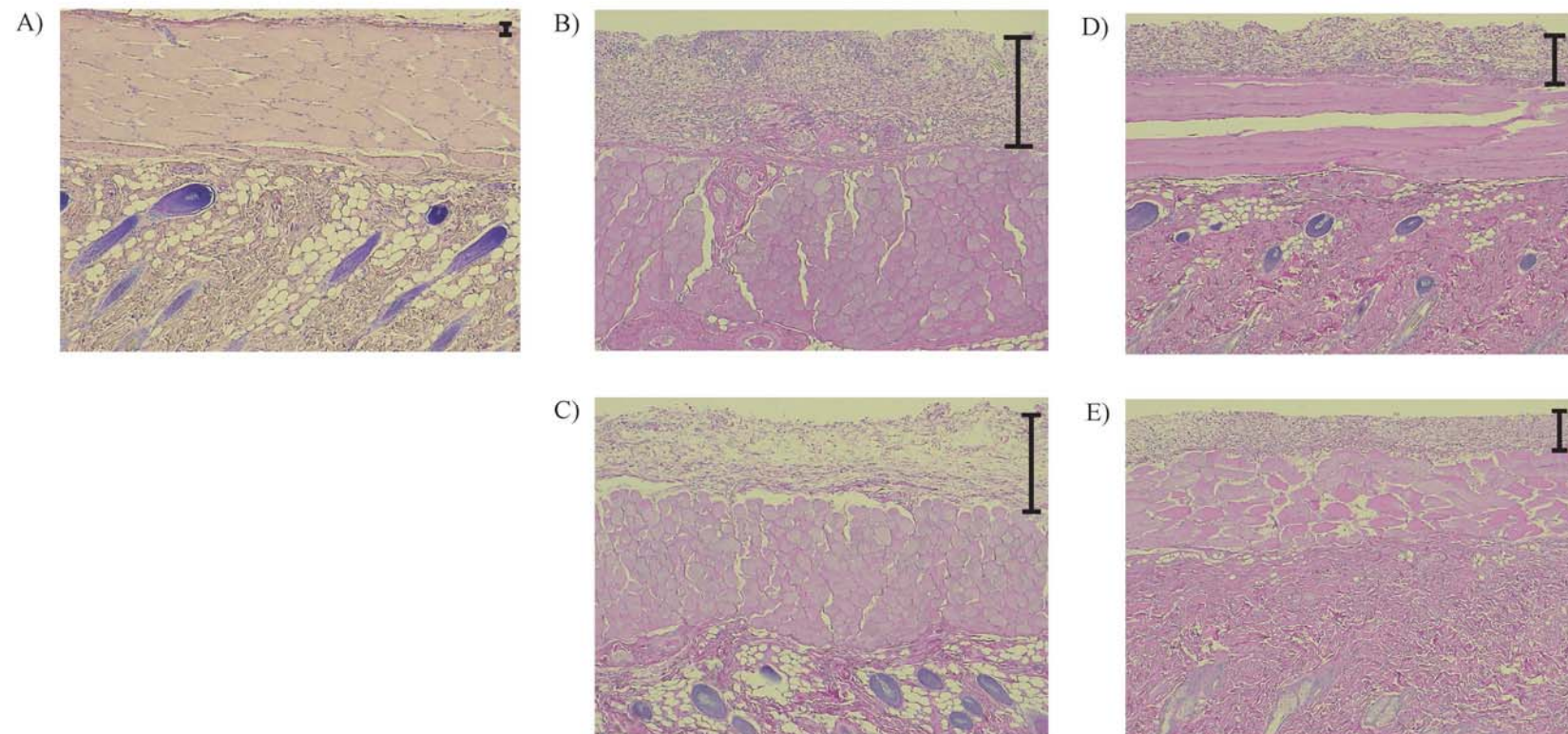




Figure 3

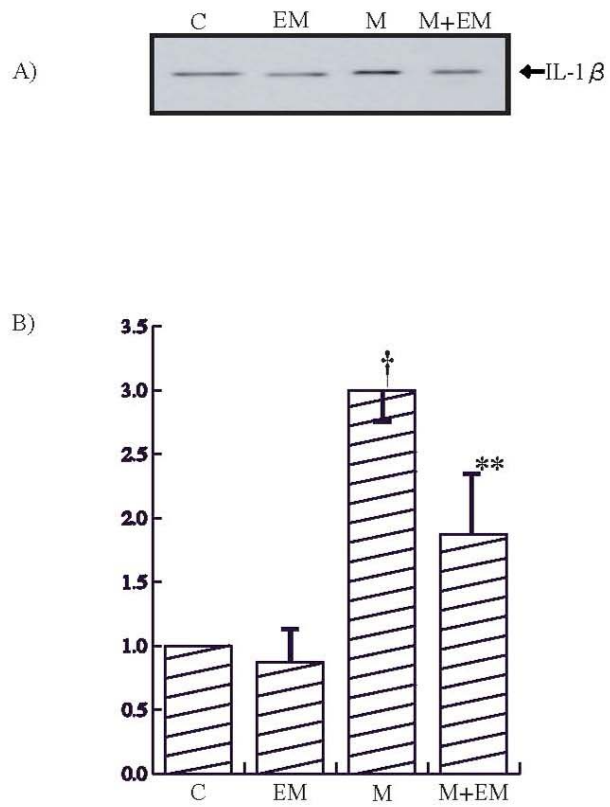


Figure 4

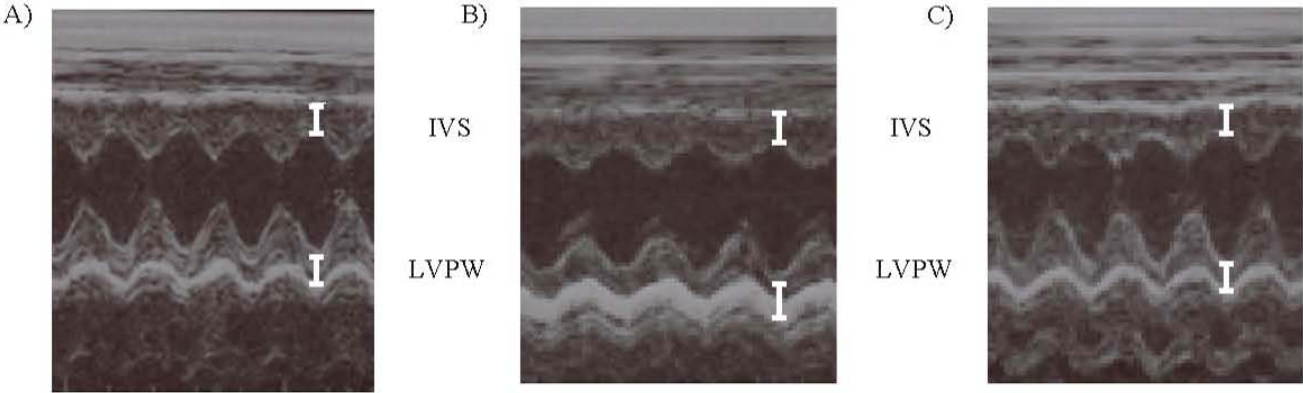




Table 1. Organ weights and cardiac pathology

			BW	HW/BW	LuW/BW	LiW/BW	Cardiac Pathology (0~+5)	
			(n)	(g)	( $\times 10^{-3}$ )	( $\times 10^{-3}$ )	( $\times 10^{-3}$ )	Infiltration      Necrosis
Immunized Protocol								
Control		15	261 $\pm$ 20	4.5 $\pm$ 0.4	4.3 $\pm$ 0.3	42.3 $\pm$ 4.0	3.2 $\pm$ 0.5	2.8 $\pm$ 0.7
Erythromycin	3.3mg/kg/day	15	270 $\pm$ 17	4.3 $\pm$ 0.5	4.1 $\pm$ 0.5	42.2 $\pm$ 3.8	2.7 $\pm$ 0.9	2.5 $\pm$ 0.9
	33mg/kg/day	10	258 $\pm$ 10	3.8 $\pm$ 0.4*	4.1 $\pm$ 0.3	41.6 $\pm$ 2.8	1.8 $\pm$ 0.7*	1.9 $\pm$ 0.4*
Unimmunized Protocol								
Control		5	260 $\pm$ 30	3.8 $\pm$ 0.2	4.3 $\pm$ 0.3	42.0 $\pm$ 4.0	---	---
Erythromycin	3.3mg/kg/day	5	258 $\pm$ 25	3.7 $\pm$ 0.4	4.4 $\pm$ 0.4	43.4 $\pm$ 3.8	---	---
	33mg/kg/day	5	238 $\pm$ 21	3.9 $\pm$ 0.2	4.0 $\pm$ 0.3	39.0 $\pm$ 4.2	---	---

(mean $\pm$ SD)

HW/BW ratio was lower in the erythromycin group (33mg/kg/day) compared to the control. Semi-quantitative histological grades for infiltration and necrosis were significantly lower in the erythromycin group (33mg/kg/day) compared to the control. BW=body weight, HW=heart weight, LuW=lung weight, LiW=liver weight, \*P < 0.05 v Control.

Table 2. Immunohistochemistry, chemotaxis and DHE staining

		Positive Cells (%)			Leukocyte Chemotaxis	Ethidium Fluorescence
		ED1 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	(× 10 <sup>5</sup> )	(units)
Control		20.6±4.5	15.0±5.0	7.5±3.8	25.5±3.2	1.00±0.09
Erythromycin	3.3mg/kg/day	12.7±3.6*	7.8±3.2*	4.4±2.6	18.3±3.5*	0.82±0.05*
	33mg/kg/day	5.8±5.8**	4.8±8.6**	3.8±3.0*	12.4±4.5**	0.79±0.08**

(mean ± SD)

The percentages of ED1<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> positive cells in the erythromycin group (3.3mg/kg/day and 33mg/kg/day) were lower than in the untreated (control) mice.

The number of leukocytes exudated into air pouch in unimmunized groups (control group and erythromycin group) ranged from 16.5±3.5× 10<sup>5</sup>.

For leukocyte chemotaxis and ethidium fluorescence assay, each trial was independently performed 4-5 times.

\*P < 0.05, \*\*P<0.01 vs Control.

Table 3. Echocardiographic data

		(n)	HR (hearts/min)	LVDd (mm)	EF (%)	IVST (mm)	LVPWT (mm)
Immunized Protocol							
Control		8	407±16	6.5±0.3	83.1±3.6	2.0±0.6	2.1±0.3
Erythromycin	33mg/kg/day	8	402±30	6.3±0.4	82.1±4.5	1.9±0.5	1.6±0.2*
Unimmunized Protocol							
Control		5	405±5	6.2±0.2	86.6±10.0	1.9±0.3	1.4±0.4
Erythromycin	33mg/kg/day	5	446±7	6.0±0.2	84.6±7.6	2.0±0.5	1.5±0.4

EM treatment suppressed the increase of left ventricular LVPWT compared with the untreated groups. EF=ejection fraction, HR=heart rate, IVST=interventricular septum thickness, LVDd=left ventricular end-diastolic dimension, LVPWT=left ventricular posterior wall thickness. \*P<0.05 vs Control.